Interferon- α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator

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The interaction of interferon- α (IFN- α) with a specific cell-surface receptor elicits physiological changes that rely on rapid transcriptional activation of a group of IFN- α -stimulated genes (ISGs). The IFN-stimulated response element (ISRE), a conserved regulatory element of all ISGs, is the target for transcriptional activation by the positive regulator IFN-stimulated gene factor-3 (ISGF3). We reported previously that post-translational activation of ISGF3 in the cytoplasm of IFN- α -treated cells requires two cytoplasmic activities (ISGF3 α and ISGF3 γ) to produce an ISRE-binding complex that accumulates in the nucleus. In this study, we show that these activities are actually distinct subunits of the ISGF3 complex, which associate through noncovalent interaction. Sedimentation analysis, protein renaturation, and photoaffinity cross-linking of enriched preparations of cytoplasmic ISGF3 α and ISGF3 γ and of nuclear ISGF3 demonstrated that ISGF3 γ was a 48-kD polypeptide with intrinsic, low-affinity DNA-binding activity. Four polypeptides of 48, 84, 91, and 113 kD bound to the ISRE *in vitro*; the larger three polypeptides most likely compose the ISGF3 α component. These ISGF3 α polypeptides were unable to bind DNA alone but formed a DNA-binding complex in conjunction with ISGF3 γ . The resulting heteromeric complex had the same ISRE-binding specificity as the individual ISGF3 γ polypeptide but ~25-fold higher affinity. Whereas ISGF3 γ partitioned between the cytoplasm and nucleus in unstimulated cells, ISGF3 α was stimulated to translocate to the nucleus only following IFN- α treatment, resulting in preferential nuclear accumulation of both ISGF3 α and ISGF3 γ as a stable ISGF3-ISRE complex. This regulated nuclear translocation of an activated transcription factor subunit maintained the specificity and rapidity of the IFN- α signaling pathway.

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The interferon (IFN) system provides a useful model for studying the mechanisms by which eukaryotic cells transduce complex extracellular signals resulting in modulation of specific gene expression. Type I interferons (IFN- α/β) interact with a specific cell-surface receptor present on nearly all human cell types. The major physiological responses to IFNs are cessation of cell growth and inhibition of viral replication, effects that depend on *de novo* synthesis of dozens of IFN-induced gene products (for review see De Maeyer and De Maeyer-Guignard 1988).

The transcriptional response of a group of IFN- α -stimulated genes (ISGs), an initial event in the response to IFN- α , has been described. Activation is rapid, detectable within 10 min of exposure to IFN- α , and of large

magnitude with transcriptional increases >50-fold within 1 hr of treatment (Friedman et al. 1984; Larner et al. 1984, 1986; Friedman and Stark 1985). Activation of transcription occurs in the absence of new protein synthesis, implying a role for post-translational modification of a preexisting cellular protein(s) in transcriptional induction. In addition, the response is transient, returning to basal transcription levels within 8–15 hr in most cell lines. The transient aspect of the response, or down-regulation, is dependent on new protein synthesis, and the inclusion of protein synthesis inhibitors during IFN- α treatment results in prolonged transcription (Friedman et al. 1984; Larner et al. 1984, 1986; Levy et al. 1986).

Analysis of ISG promoters has identified a *cis*-regulatory target, the interferon-stimulated response element (ISRE) (Friedman and Stark 1985; Israel et al. 1986; Vogel et al. 1986; Reich et al. 1987; Sugita et al. 1987; Wathlet et al. 1987; Levy et al. 1988; Porter et al. 1988),

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which is both necessary and sufficient for ISG transcriptional stimulation (Cohen et al. 1988; Reich and Darnell 1989), and is the ultimate target for the IFN- α signaling pathway. We and others have identified nuclear proteins that bind specifically to the ISRE (Cohen et al. 1988; Levy et al. 1988; Porter et al. 1988; Rutherford et al. 1988; Shirayoshi et al. 1988), one of which (ISGF3) is the primary transcriptional activator of ISGs (Kessler et al. 1988a; Levy et al. 1988, 1989; Dale et al. 1989b). The role of ISGF3 as the transcriptional activator has been confirmed through analysis of its binding to an extensive set of point mutations of the ISRE (Kessler et al. 1988a), measurement of the kinetics of its activation (Levy et al. 1988, 1989), absence of protein synthesis requirements (Levy et al. 1988), and correlation of its activation with ISG transcription in a variety of responsive and resistant cell lines (Kessler et al. 1988b). ISGF3 is distinct from several other proteins that bind the ISRE. In particular, it appears to be unrelated to ISGF2 (equivalent to the mouse protein IRF-1 described by Fujita et al. 1988), an IFN-inducible ISRE-binding protein that plays no role in the primary induction of ISGs (Pine et al. 1990).

Studies with agents that perturb known intracellular signaling systems have failed to implicate such messengers as components of the IFN- α signal transduction apparatus (Tamm et al. 1987; Lew et al. 1989). Furthermore, the recent cloning of a subunit of the IFN- α receptor revealed a noncanonical transmembrane protein (Uzé et al. 1990), providing no clues about the earliest events of IFN- α signal transduction. However, ISGF3 is rapidly activated in the cytoplasm of IFN- α -treated cells prior to its translocation to the nucleus (Dale et al. 1989b; Levy et al. 1989), pointing to a central role for ISGF3 in early steps in signal transduction, as well as in subsequent transcriptional activation. Cytoplasmic ISGF3 can be detected within 1 min of stimulation, whereas nuclear ISGF3 is undetectable before 5 min of treatment. Furthermore, Dale et al. (1989a) have shown that ISGF3 can be stimulated in enucleated cells, demonstrating that ISGF3 is activated in the cytoplasm. Development of an *in vitro* system for cytoplasmic activation revealed two distinct activities which when mixed form ISGF3 (Levy et al. 1989). One activity (termed ISGF3 γ) could be detected in both untreated and IFN- α -treated cell cytoplasm, while the other (termed ISGF3 α) was detected only following IFN- α treatment. These two activities had distinct characteristics: whereas ISGF3 α was detected only following IFN- α treatment (hence, its name), ISGF3 γ was induced by IFN- γ treatment and required both RNA transcription and protein synthesis for its increased abundance following IFN- γ treatment (Levy et al. 1990). In addition, ISGF3 γ was sensitive to alkylation by *N*-ethyl maleimide (NEM) whereas ISGF3 α was not. The parameters necessary for *in vitro* formation of ISGF3 led us to suggest that it resulted from stoichiometric interactions between two components.

We have now examined the molecular composition and regulation of ISGF3. ISGF3 was found to be a nonco-

valent complex separable into two distinct subunits that correlated with ISGF3 α and ISGF3 γ activity. The ISGF3 γ subunit was a 48-kD DNA-binding protein with high specificity but low affinity for the ISRE. ISGF3 α showed no DNA-binding activity and was composed of three larger polypeptides. These ISGF3 α proteins accumulated to high levels in nuclei following IFN- α treatment through a process inhibited by NaF. Activated ISGF3 α modulated the DNA-binding affinity of ISGF3 γ to form the high-affinity ISGF3 complex on the ISRE.

Results

ISGF3 γ is a novel DNA-binding activity with specificity for the ISRE

ISGF3 is detected in cell extracts by using gel mobility-shift assays based on two criteria: characteristic mobility of the protein-DNA complex and a binding specificity that distinguishes between the full-length ISRE and mutant sequences containing only the central core region required for binding other ISRE-binding proteins (Kessler et al. 1988a). This factor is detected only in extracts of HeLa cells that were treated previously with IFN- α (Levy et al. 1988, 1989) but is 10-fold more abundant in extracts of cells that have been primed with IFN- γ prior to exposure to IFN- α (Levy et al. 1990). ISGF3 α and ISGF3 γ are complementing cytoplasmic activities that produce the ISGF3 complex when mixed *in vitro* (Levy et al. 1989). We have fractionated these activities individually from separate cytoplasmic extracts and compared these activities with ISGF3 isolated from nuclear extracts of stimulated cells. Cytoplasmic extracts were prepared from IFN- γ -treated HeLa S3 cells, containing high levels of ISGF3 γ activity but no mature ISGF3 because the cells were never exposed to IFN- α . Alternatively, cytoplasmic extracts prepared from IFN- α -treated cells were alkylated *in vitro* with NEM to inactivate ISGF3 γ and thus contained only ISGF3 α activity (Levy et al. 1989).

Using a synthetic oligonucleotide containing an ISRE sequence rather than the promoter fragment used previously as probe in gel-shift experiments, we observed an ISRE-binding activity in cytoplasmic extracts of cells not treated with IFN- α (Fig. 1A). This novel activity (labeled ISGF3 γ) displayed a distinct mobility and yet the same DNA-binding specificity as ISGF3 in that the protein-DNA complex was competed by excess unlabeled full-length ISRE oligonucleotides (lane 3) but not by heterologous (lane 1) or core (lane 2) oligonucleotides, and it protected a footprint identical to ISGF3 (not shown). This binding factor was dramatically induced in response to IFN- γ in both cytoplasm (lanes 7–9) and nuclei (lanes 10–12). It was distinct from the constitutive and inducible ISRE-binding factors, ISGF1 and ISGF2 (Levy et al. 1988), which displayed different electrophoretic mobilities, different affinities for oligonucleotide competitors, and were detected only in extracts of nuclei (lanes 10–12). In addition, this novel DNA-binding activity was sensitive to alkylation by NEM (not shown).

The NEM sensitivity, binding specificity, cytoplasmic

Figure 1. ISGF3 γ displays intrinsic DNA-binding ability. (A) Binding of cytoplasmic (C) or nuclear (N) factors from untreated (lanes 1–6) or IFN- γ -treated (lanes 7–12) HeLa cells to an ISRE oligonucleotide. Competing unlabeled heterologous (ns oligo), core, or ISRE oligonucleotide was included in the binding reactions as indicated. The mobilities of protein–DNA complexes composed of ISGF1, ISGF2, and ISGF3 γ are indicated. ISGF1 and ISGF2 were observed only in nuclear extracts, and binding of these proteins was competed by both core and ISRE oligonucleotides (Kessler et al. 1988a). Slower mobility complexes in lane 10 also competed by both core and ISRE oligonucleotides are additional forms of ISGF2 (Pine et al. 1990). ISGF3 γ was seen in both cytoplasm and nuclei and was highly inducible by IFN- γ (Levy et al. 1990). (B) Partially purified preparations of ISGF3 α and ISGF3 γ from cytoplasm (c) and of ISGF3 from nuclei (n) were mixed with labeled ISRE DNA in the presence of excess competitors, as indicated. ISGF3 α preparations (lanes 1, 5, 9) contained no DNA-binding activity prior to mixing with ISGF3 γ to form ISGF3 (lanes 3, 7, 11), which displayed identical mobility and sequence specificity as nuclear ISGF3 (lanes 4, 8, 12). ISGF3 γ binding to DNA (lane 2) was competed only by full-length ISRE oligonucleotides (lane 10) and was depleted on addition of ISGF3 α , coincident with formation of ISGF3 (cf. lanes 2 and 3).

localization and IFN- γ inducibility of this DNA-binding activity prompted us to consider its relationship to previously described ISGF3 γ . ISGF3 α and ISGF3 γ from cytoplasmic extracts were separately purified >2000-fold by biochemical fractionation (see Methods). Column fractions were assayed for activity by mixing with unfractionated HeLa cell extracts containing the complementing activity and measuring the resulting ISGF3 levels. These highly enriched fractions were sufficient to reconstitute ISGF3 when mixed with each other, suggesting that no additional components, besides ISGF3 α and ISGF3 γ , that might have been present in unfractionated extracts were necessary for ISGF3 formation (Fig. 1B, lane 3). The enriched preparation of ISGF3 α contained no detectable DNA-binding activity (lane 1). On the other hand, fractionated ISGF3 γ (lane 2) displayed an activity with the same gel-shift mobility as the novel IFN- γ -inducible DNA-binding activity observed in crude extracts (cf. with Fig. 1A). This DNA-binding activity copurified precisely with ISGF3 γ activity through every chromatographic step (not shown). Furthermore, addition of ISGF3 α to this preparation of ISGF3 γ caused a significant depletion of the IFN- γ -inducible gel-shift

complex coincident with the appearance of the slower migrating ISGF3 complex (cf. lanes 2 and 3). Highly enriched fractions of ISGF3 isolated from nuclei by ISRE affinity chromatography also contained this DNA-binding activity in addition to ISGF3 (lane 4). DNA-binding specificity of these enriched preparations was restricted to the ISRE and was identical to nuclear ISGF3, as judged by competitive gel-shift analysis (lanes 5–12), suggesting that ISGF3 γ is a DNA-binding component of ISGF3. The gel-shift mobility of ISGF3 isolated from nuclei was slower than ISGF3 created by mixing cytoplasmic ISGF3 α and ISGF3 γ in vitro (Fig. 1B). Cytoplasmic ISGF3 often appeared as a doublet (see also Fig. 2B). This doublet appeared to be a distinct characteristic of the cytoplasmic, rather than nuclear, ISGF3 α component.

ISGF3 can be separated into its constituent components

Nuclear ISGF3 was found to contain separable ISGF3 α and ISGF3 γ activities when fractionated on a 15–30% glycerol gradient. Although a substantial amount of ISGF3 was loaded onto the gradient (Fig. 2A, lane L), no

Figure 2. Glycerol gradient sedimentation fractionates ISGF3 into separate ISGF3 α and ISGF3 γ activities. Nuclear ISGF3 was fractionated by glycerol gradient centrifugation, and each fraction (1-15) was assayed for activity by gel shift either before (A) or after addition of cytoplasmic ISGF3 α (B) or ISGF3 γ (C). Note that although no ISGF3 was detected in the direct assay, ISGF3 γ was detected in fractions 4-6. After mixing with ISGF3 α , this ISGF3 γ formed ISGF3 (B). Mixing with ISGF3 γ (C) formed ISGF3 from material in fractions 9 and 10, signifying the presence of ISGF3 α fractionating at this position in the gradient. Portions of each fraction were analyzed by 7.5% SDS-PAGE, followed by silver staining (Wray et al. 1981) (D). Note that fractions 4 and 5 contained a 48-kD polypeptide corresponding to ISGF3 γ . Many polypeptides cofractionated with ISGF3 α activity in fractions 9 and 10, some of which had nonspecific DNA-binding activity, accounting for the gel-shift complexes with mobilities intermediate between ISGF3 and ISGF3 γ .

ISGF3 was recovered in the gradient fractions (lanes 1-15). Instead, free ISGF3 γ was detected in fractions 4-6. The other gel-shift complexes detected were due to nonspecific DNA-binding activities. One possible explanation for this result is that ISGF3 was an unstable complex (under these conditions) composed of distinctly sedimenting subunits. To test for such separated activities, gradient fractions were mixed with ISGF3 α (Fig. 2B) or ISGF3 γ (Fig. 2C) enriched from cytoplasmic extracts. ISGF3 activity was readily reconstituted by this *in vitro* mixing. Addition of ISGF3 α to fractions 4-6 drove ISGF3 γ into the slower ISGF3 gel-shift complex (Fig. 2B),

whereas complementary mixes with ISGF3 γ revealed a distinct, nonoverlapping peak of ISGF3 α activity in fractions 9-10 (Fig. 2C). These data indicated that ISGF3 α sedimented with a relative molecular mass of ~80 kD, whereas ISGF3 γ sedimented as an ~50-kD protein.

The polypeptide composition of gradient fractions was resolved by 7.5% SDS-PAGE and stained with silver. The starting fraction consisted of four highly abundant proteins and many additional polypeptides (Fig. 2D, lane L). A single polypeptide of ~48 kD, corresponding to one of the less abundant proteins in the starting fraction, precisely cosedimented with ISGF3 γ activity (lanes

4–6), and was tentatively identified as ISGF3 γ (see below). Identification of potential ISGF3 α polypeptides was considerably more complicated. Several proteins were detected in gradient fractions containing peak ISGF3 α activity (lanes 9 and 10). Comparison of the sedimentation pattern of these proteins throughout the gradient allowed tentative identification of two highly abundant polypeptides, four additional major polypeptides, and numerous minor polypeptides that precisely cosedimented with ISGF3 α activity.

The recovery of distinct peaks of ISGF3 α and ISGF3 γ activity suggested that mature ISGF3 was composed of these two subunits. When ISGF3 α and ISGF3 γ peak activity fractions from a glycerol gradient were mixed with each other rather than with cytoplasmic fractions, ISGF3 was again recovered (Fig. 3A), indicating that proteins sedimenting in these two size ranges were sufficient to reconstitute ISGF3. Likewise, enriched preparations of ISGF3 γ and ISGF3 α derived from cytoplasmic extracts were separately fractionated by sedimentation. Each activity cosedimented with its nuclear cognate, suggesting that no gross differences existed between the cytoplasmic and nuclear forms of ISGF3 subunits (not shown).

Why did ISGF3 separate into its constituent subunits during centrifugal sedimentation? A variation on the gradient experiment was performed in which nuclear ISGF3 was incubated with a radiolabeled ISRE probe prior to sedimentation analysis (Fig. 3B). Under these conditions, ISGF3 and the ISRE cosedimented as a complex with an apparent molecular mass of 250–350 kD. No free ISGF3 α or ISGF3 γ was detected in the lighter fractions of the gradient by mixing with complementing activities (not shown). Control experiments with an unrelated oligonucleotide probe did not show stabilization of the ISGF3 complex. These data suggested that although ISGF3 α and ISGF3 γ may not associate stably, a ternary complex could be formed between these ISGF3 subunits and the ISRE. In addition, the native ISGF3–DNA complex appeared to be larger than a simple 1 : 1 association of ~50-kD ISGF3 γ and ~80-kD ISGF3 α subunits.

Polypeptide composition of ISGF3

To characterize further the sizes of individual polypeptides comprising ISGF3, fractions of cytoplasmic ISGF3 γ or ISGF3 α were resolved by SDS-PAGE, and protein eluted from gel slices was renatured and assayed by gel shift for the ability to form ISGF3 after mixing with complementing ISGF3 α or ISGF3 γ . Protein recovered from the 42- to 55-kD range produced ISGF3 when mixed with ISGF3 α (Fig. 4, lane 7), consistent with sedimentation analysis (see Fig. 2), indicating that ISGF3 γ was a polypeptide of ~50 kD. No detectable ISGF3 α activity was recovered by the same renaturation procedure from any region of the SDS gel (data not shown). This negative result may indicate that recovery of ISGF3 α activity was exceedingly low under conditions used, or, alternatively, that ISGF3 α consisted of more than a single polypeptide and that the necessary subunits were not present in any single gel slice (see below).

Photoaffinity cross-linking was used to identify proteins in close proximity to the ISRE. A uniformly 32 P-labeled ISRE oligonucleotide containing 5-azido-deoxyuridine (Evans et al. 1986; Evans and Haley 1987) was incubated with appropriate fractions, and DNA–protein complexes were resolved by gel shift (Fig. 5A). The intact gel was exposed to ultraviolet light, and ISGF3 γ – and ISGF3–ISRE complexes were excised. Cross-linked products were electroeluted directly into 8% SDS-PAGE, and radiolabeled polypeptides were identified following autoradiography (Fig. 5B). A single labeled polypeptide of ~50 kD was detected from the ISGF3 γ –ISRE gel-shift complex, a size consistent with both sedimentation (Fig. 2) and renaturation from SDS-PAGE (Fig. 4) analyses after adjustment for the presence of linked oligonucleotide (Fig. 5B, lanes 1 and 3). As expected, a polypeptide of identical mobility was detected from the ISGF3–DNA complex, from both nuclear ISGF3 and ISGF3 reconstituted *in vitro* from cytoplasmic activities. In addition, higher-molecular-weight polypeptides not present in the ISGF3 γ –DNA complex were detected in the ISGF3 complex (lanes 2 and 4). Three proteins in the range of 80–120 kD (after adjustment) plus a fourth very

Figure 3. Interaction of ISGF3 components is stabilized by DNA. (A) Pooled fractions of separated ISGF3 α and ISGF3 γ from a glycerol gradient were assayed for DNA-binding activity alone (lanes 2 and 3) and following mixing *in vitro* with each other (lanes 4–6). Binding specificity of the ISGF3 formed from these fractions was tested by competition with heterologous (lane 4), core (lane 5), or ISRE oligonucleotides (lane 6). (B) ISGF3–ISRE complexes formed *in vitro* between nuclear ISGF3 and labeled ISRE DNA were fractionated by glycerol gradient sedimentation. Each fraction (1–23) was assayed directly for the presence of complexes by gel shift. Positions of ISGF3 and free ISRE are indicated, as are the migrations of molecular weight standards from a parallel gradient.

Figure 4. SDS-PAGE fractionation and renaturation of ISGF3 γ . ISGF3 γ purified from cytoplasm was fractionated on 10% SDS-PAGE. Protein recovered from individual gel slices corresponding to marker proteins of the indicated molecular weights was renatured (Briggs et al. 1986) and analyzed for ISGF3 γ activity by mixing with cytoplasmic ISGF3 α . ISGF3 γ was recovered in a single gel slice (lane 7) corresponding to 42–55 kD.

large species (>200 kD) were specifically cross-linked and therefore presumably were in close proximity with the ISRE. Once again, no gross differences between cytoplasmic and nuclear fractions were apparent (cf. lane 1 with 3 and lane 2 with 4). The additional higher-molecular-weight proteins detected in the ISGF3 complex may represent ISGF3 α polypeptides brought into close proximity of DNA through interaction with ISGF3 γ bound at the ISRE. However, the submolar representation of these higher-molecular-weight polypeptides suggests that they were inefficiently cross-linked to DNA and therefore probably not making direct, base-specific contacts with DNA.

Direct identification of the polypeptide subunits of ISGF3 was possible through a preparative gel-shift analysis. Large-scale DNA-binding reactions consisting of nuclear ISGF3 incubated with an ISRE or unrelated radiolabeled oligonucleotide probe were resolved by electrophoresis. ISGF3 and a parallel region from a control lane were excised, and polypeptides were directly electroeluted into an 8% SDS gel and stained with silver (Fig. 6). Several polypeptides were recovered from the gel slice containing ISGF3 bound to the ISRE, three corresponding to proteins present in high abundance in the starting fraction and four corresponding to less abundant proteins (lane 3). A parallel region from a control reaction, using a heterologous oligonucleotide probe, contained only the three high-abundance proteins (lane 4), indicating that the four low-abundance proteins from the starting fraction were specifically enriched in the ISRE complex. Sizes of the four specific proteins were consistent with other analyses of ISGF3 α and ISGF3 γ . A 48-kD protein was detected, corresponding to ISGF3 γ as

determined by renaturation, cross-linking, and sedimentation. The other three proteins (84, 91, and 113 kD) would be candidates for ISGF3 α polypeptides. These sizes are consistent with the ISGF3 α sedimentation profile, with the sedimentation of the ISGF3–ISRE complex, with the higher-molecular-weight photoaffinity cross-linked products, and with the failure to renature ISGF3 α from a discrete size fraction of an SDS gel. Only a 48-kD polypeptide was recovered when the ISGF3 γ –ISRE gel-shift complex was analyzed by this procedure (not shown).

Figure 5. Photoaffinity cross-linking of ISGF3 polypeptides. (A) Nuclear ISGF3 (lane 1) and cytoplasmic ISGF3 α (lane 2), ISGF3 γ (lane 3), and ISGF3 formed in vitro (lane 4) were separated on mobility-shift gels following incubation with 32 P-labeled ISRE DNA containing azido-deoxyuridine residues. The intact gel was exposed to UV light, and following autoradiography, protein–DNA complexes were excised and electroeluted into 8% SDS-PAGE (B). ISGF3 γ from the nuclear ISGF3 preparation was loaded in lane 1, nuclear ISGF3 in lane 2, cytoplasmic ISGF3 γ in lane 3, and in vitro-formed cytoplasmic ISGF3 in lane 4 (arrows). Specifically labeled polypeptides from the ISGF3 complex are indicated by marks on the right. These products were not detected when free probe was subjected to similar analysis (not shown).

Figure 6. Preparative gel-shift analysis of ISGF3 identifies four proteins. Nuclear ISGF3 was fractionated by gel shift, using an ISRE or heterologous oligonucleotide probe. Gel slices corresponding to the ISGF3 complex or the same region from a parallel control lane were excised and electroeluted into an SDS gel. (Lane 1) Molecular weight markers; (lane 2) the ISGF3 fraction used; (lanes 3 and 4) proteins recovered from the ISGF3 gel-shift complex or control lane, respectively. Specifically recovered proteins displayed mobilities consistent with sizes of 113, 91, 84, and 48 kDa, as indicated.

Low-affinity interactions between ISGF3 γ and the ISRE are stabilized by ISGF3 α

The stability of the protein–DNA complexes was directly examined by dissociation rate measurements. The ISGF3 complex or the ISGF3 γ subunit was preincubated with the ISRE under standard DNA-binding conditions for 20 min at room temperature, a period sufficient for the interaction to reach equilibrium (Levy et al. 1989). A 500-fold molar excess of unlabeled ISRE oligonucleotide was added to the mixture, and the stability of the preformed protein–DNA complexes was determined over time. At serial time points after addition of competitor, aliquots were removed and loaded directly onto a running polyacrylamide gel (Fig. 7A). Half-lives of the complexes were calculated assuming first-order dissociation kinetics (Fig. 7B). Dissociation of the ISGF3–ISRE complex was slow, having a half-life of ~ 53 min (the range of four independent measurements was 40–68 min). The ISGF3 γ –ISRE interaction, however, was strikingly less stable, with a half-life of <2 min (the range of three determinations was 1.1–2.3 min). Essentially identical results were obtained for nuclear and cytoplasmic preparations of ISGF3 and ISGF3 γ , either when bound to a 130-bp ISG15 promoter DNA fragment containing the ISRE or when bound to a 32-bp synthetic ISRE probe. The ~ 25 -fold lower dissociation rate of ISGF3 indicated a substantially higher affinity of the complex for the ISRE over that of the putative DNA-binding component alone. These results suggest that ISGF3 is assembled from a regulatory subunit, displaying no intrinsic DNA-

binding activity (ISGF3 α), in an allosteric complex with a low-affinity DNA-binding chain (ISGF3 γ), yielding a high-affinity interaction with the ISRE.

Regulated nuclear translocation of ISGF3 α

The activation of ISGF3 in the cytoplasm of IFN- α -stimulated cells and its subsequent appearance in the nucleus implied nuclear translocation of one or both of the components of ISGF3. Quantitation of the cytoplasmic and nuclear levels of ISGF3 components provided evidence for independent nuclear translocation of ISGF3 α . Extracts prepared from untreated or IFN- α -stimulated cells were assayed for ISGF3 activity and, for total levels of ISGF3 α and ISGF3 γ , by addition of excess enriched cytoplasmic components (Fig. 8A). Addition of ISGF3 γ to induced extracts resulted in a marked increase in ISGF3 activity, suggesting the presence of excess ISGF3 α over the level of ISGF3 γ in both the cytoplasm and nucleus of stimulated cells (cf. Fig. 8A, lanes 2 and 6 and lanes 4 and 8; see also Fig. 8B, lane 8). Addition of ISGF3 α , on the other hand, although detecting constitutive cytoplasmic ISGF3 γ (lane 10), did not enhance the level of nuclear ISGF3 over that obtained without mixing (cf. lanes 4 and 13). These observations indicated that ISGF3 α was activated in cells in excess of the level of ISGF3 γ and that it accumulated preferentially in nuclei also in excess. Therefore, ISGF3 α was capable of nuclear translocation presumably independent of its association with ISGF3 γ . In contrast, nuclear ISGF3 γ levels corresponded to total nuclear ISGF3. Accumulation of substantial nuclear ISGF3 γ was dependent on the presence of ISGF3 α , probably through stabilization of its participation in an ISGF3 complex with the ISRE.

Further evidence in support of a role for regulated nuclear translocation of ISGF3 α was obtained through the use of NaF, an inhibitor of ISG induction. We reported previously that a partial inhibition of ISG induction by IFN- α was caused by a reduced nuclear accumulation of ISGF3 (Levy et al. 1989). A modification of this protocol resulted in quantitative inhibition of nuclear accumulation of ISGF3 (Fig. 8B). NaF pretreatment of cells for 1 hr followed by IFN- α stimulation resulted in normal total ISGF3 levels, but $>95\%$ of the activity was retained in the cytoplasm (lane 3), with virtually no ISGF3 appearing in nuclei (lane 6). To explore the level at which NaF blocked nuclear accumulation of ISGF3, cytoplasmic and nuclear extracts of NaF pretreated or control IFN- α -treated cells were assayed for ISGF3 α and ISGF3 γ . Exclusion of ISGF3 from nuclei in NaF-treated cells was reflected in extremely low levels of nuclear ISGF3 α (lane 9). ISGF3 γ levels were unperturbed (data not shown). Therefore, inhibition by NaF appeared to act specifically at the level of ISGF3 α nuclear translocation, an event essential for nuclear accumulation of ISGF3.

Discussion

We have presented evidence that ISGF3 is a multisubunit complex composed of ISGF3 α and ISGF3 γ , sup-

Figure 7. ISGF3 forms a much more stable complex with ISRE DNA than does ISGF3 γ . (A) Protein-DNA complexes were formed between nuclear ISGF3 (lanes 1–12), ISGF3 formed in vitro from cytoplasmic ISGF3 α and ISGF3 γ (lanes 13–25), or ISGF3 γ alone (lanes 26–37) and a labeled ISG15 promoter fragment containing the ISRE. Stability of these complexes was determined by measuring the amount of residual labeled DNA in a specific complex at serial times following addition of unlabeled DNA oligonucleotides. Portions of each binding reaction were removed at the indicated times (min) and loaded directly onto a running mobility-shift gel. Samples were loaded at differing times throughout the experiment and were therefore electrophoresed for different lengths of time, resulting in somewhat different mobilities of the ISGF3 complex and of the free probe for different samples. (B) The autoradiograms shown in A were quantitated by laser densitometry. Log percent of the density from starting complexes was plotted against time and fitted to a simple line by least-squares regression.

porting our previous proposition that it was formed from stoichiometric association of distinct subunit activities (Levy et al. 1989). Glycerol gradient sedimentation demonstrated that both ISGF3 α and ISGF3 γ were present as constituents of nuclear ISGF3; however, the association of these two activities with each other to form ISGF3 appeared to be stable only when bound to DNA. Analyses of partially purified fractions of cytoplasmic ISGF3 α and ISGF3 γ as well as of nuclear ISGF3 by photoaffinity labeling and polyacrylamide gel electrophoresis, demonstrated the distinct characteristics of these individual activities and revealed a multicomponent nature for ISGF3 α . ISGF3 α and ISGF3 γ , either fractionated from cytoplasm or recovered from enriched fractions of nuclear ISGF3, were sufficient to reconstitute ISGF3 in vitro without requiring additional cellular proteins. Interestingly, ISGF3 γ was found to be a novel ISRE-

binding protein present in both cytoplasm and nuclei of unstimulated cells. Its low-affinity binding to DNA was stabilized by interaction with ISGF3 α .

The molecular composition of ISGF3 appeared to be heteromeric, consisting of a 48-kD DNA-binding chain (ISGF3 γ) and probably multiple regulatory proteins of higher molecular weight comprising ISGF3 α . Four proteins were specifically retained in an ISGF3 gel-shift complex. One of those polypeptides was 48 kD, identical to ISGF3 γ ; the others (84, 91, and 113 kD) are probably constituents of ISGF3 α .

ISGF3 α was an early target in IFN- α signaling (Fig. 9). Its activation was presumably mediated by an as yet unidentified post-translational modification of one or more of its constituent polypeptide chains. ISGF3 α can thus be considered the regulatory subunit of ISGF3. Activation of ISGF3 α stimulated its nuclear translocation and

Figure 8. Regulated nuclear translocation of ISGF3. (A) ISGF3 α translocates to the nucleus in excess of the amounts of ISGF3 γ . HeLa cells were treated with IFN- α for 15 min as indicated. Cytoplasmic (C) or nuclear (N) extracts were analyzed for ISGF3 by mobility-shift directly (lanes 1–4) or following treatment with NEM and addition of cytoplasmic ISGF3 γ (lanes 5–8) or following addition of cytoplasmic ISGF3 α (lanes 10–13). Addition of ISGF3 γ formed much greater amounts of ISGF3 (lane 8) than had been detected by the direct assay (lane 4), revealing the presence of excess ISGF3 α in nuclei from IFN- α -treated cells. Addition of ISGF3 α to these samples (lane 13) did not produce more ISGF3 than had been detected directly (lane 4), indicating the lack of free ISGF3 γ in these extracts. Faster mobility gel-shift complexes in lanes 3 and 4 are due to nonspecific nuclear proteins that are sensitive to NEM (cf. with lanes 7 and 8). (B) NaF blocks nuclear accumulation of ISGF3. Cytoplasmic (lanes 1–3) and nuclear extracts (lanes 4–9) were prepared from HeLa cells before (lanes 1, 4, 7) or after treatment with IFN- α for 15 min (lanes 2, 5, 8) or after NaF for 1 hr followed by IFN- α for 15 min (lanes 3, 6, 9). Extracts were analyzed by mobility shift, using an ISRE probe either directly (lanes 1–6) or following addition of cytoplasmic ISGF3 γ (lanes 7–9).

its association with ISGF3 γ and DNA. Allosteric interactions between regulatory ISGF3 α and DNA-binding ISGF3 γ increased the affinity of the intact complex for the ISRE over that of the independent DNA-binding subunit.

Heteromeric transcriptional regulators

Combinatorial assembly of multicomponent transcription complexes from individual DNA-binding and transcriptional regulatory proteins appears to be a common theme in eukaryotic transcriptional control. Formation of multimeric protein complexes at single DNA sites has been found to enhance or reduce DNA-binding affinity, to generate novel DNA-binding affinities, and to modulate transcriptional activity of previously bound proteins. For example, CCAAT-box binding factors in the yeast *Saccharomyces cerevisiae* (Forsburg and Guarante 1989) and in mammalian cells (Chodosh et al. 1988) are composed of multiple, interacting proteins. Modulated DNA affinity through protein–protein interaction has been described for the AP-1 family of transcription factors [GCN4 in yeast and *fos*- and *jun*-related proteins in mammalian cells (for review, see Curran and Franz 1988)]. The individual subunits of these proteins display, at best, limited affinity for DNA, whereas homodimers or heterodimers, which are stable even in the absence of DNA, bind with high specificity and affinity.

In the case of ISGF3, the ISGF3 γ polypeptide binds the ISRE with low affinity whereas heteromeric association with the ISGF3 α subunits induces 25-fold greater stability for the protein–DNA complex. The low-affinity interaction between ISGF3 γ and DNA may explain its presence in both the nuclear and cytoplasmic compartments, an unexpected finding for a DNA-binding protein. Because ISGF3 α displays no innate DNA-binding activity, a probable basis for the high affinity of ISGF3 for DNA is an allosteric effect on ISGF3 γ imposed by interaction with ISGF3 α . An additional possibility would be the exposure of cryptic DNA-binding sites on one or more ISGF3 α polypeptides through interaction with ISGF3 γ . It should be noted that photoaffinity cross-linking of ISGF3 to the ISRE specifically labeled a set of

Figure 9. Model for signal transduction by IFN- α . Activation of ISGF3 α in cytoplasm of IFN- α -treated cells involves a post-translational modification. Activated ISGF3 α , composed of three polypeptides (p84, p91, p113) translocates to the nucleus where it can stabilize ISGF3 γ (p48) binding at the ISRE through allosteric conformational changes. ISGF3 γ is free to partition between cytoplasm and nuclei; association with ISGF3 α and stable binding at the ISRE causes it to accumulate in the nucleus following IFN- α treatment.

polypeptides in the same size range as putative ISGF3 α proteins, which were not seen in the ISGF3 γ -ISRE complex. However, if ISGF3 α makes additional contacts with DNA not present in the ISGF3 γ -ISRE complex, these contacts were not detected by alteration of the protected DNA sequence in footprinting experiments (S.A. Veals, unpubl.). The inefficient cross-linking of these larger polypeptides to DNA suggests that while they were brought into close proximity to DNA by ISGF3 γ , they did not make tight, sequence-specific contacts with the ISRE.

Similar interactions between DNA-binding domains and accessory proteins that modulate the overall affinity of the resulting complex have been noted for factors involved in transcription of the chicken ovalbumin gene (Tsai et al. 1987) and the human *c-fos* gene (Schroter et al. 1990). However, in these cases, there is no current evidence that these associations are differentially regulated in response to a specific signal. Analogous to the regulatory association of ISGF3 subunits, E2F, a cellular factor required for early gene expression by human adenovirus, is modulated in its affinity for DNA by association with a virally encoded E4 accessory factor in response to E1a activation (Hardy et al. 1989; Huang and Hearing 1989; Neill et al. 1990).

Subcellular compartmentalization and nuclear translocation in gene regulation

Subcellular division of eukaryotic cells into nucleus and cytoplasm opens the possibility of differential regulation through compartmentalization, as transcription factors excluded from the nucleus would be inactive (Hunt 1989). This may have particular relevance in terms of transcriptional responses to extracellular signals, because their nature requires passage through the cytoplasm. The glucocorticoid receptor may be anchored in the cytoplasm through interaction with hsp90 (Catelli et al. 1985; Sanchez et al. 1985) or other cellular proteins (Tai et al. 1986; Gasc et al. 1989) in the absence of ligand. However, the major regulation of the transcriptional stimulatory activity of the glucocorticoid receptor requires allosteric changes associated with hormone binding not involved in subcellular localization (Evans 1988; Picard et al. 1988). On the other hand, regulation of the transcription factor NF- κ B during B-cell maturation involves inducible changes that allow nuclear translocation, as well as the uncovering of DNA-binding activity (Lenardo et al. 1989). This factor is bound to a cytoplasmic inhibitory protein, I- κ B, which appears to anchor the transcription factor in the cytoplasm, as well as mask its DNA-binding ability (Baeuerle and Baltimore 1988a,b) and appears to be a substrate for protein kinases (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990). Likewise, it has recently been shown that the *Drosophila dorsal* protein regulates dorsal-ventral polarity during early embryogenesis by differential nuclear accumulation (Roth et al. 1989; Rushlow et al. 1989; Steward 1989).

Regulated nuclear localization is central to the activation of ISGF3. Intracellular signaling triggered by IFN- α binding to its cell-surface receptor targets ISGF3 α for modification in the cytoplasm. Neither the nature of ISGF3 α modification nor the intracellular events leading to its activation are yet known, but the consequence of these events is the ability of ISGF3 α to translocate to the nucleus, associate with ISGF3 γ , and assemble an active transcription complex on the ISRE. Whether release from a cytoplasmic anchor or creation or uncovering of nuclear translocation signals is the basis for this regulation is not known and must await further characterization of these proteins. However, it would appear that ISGF3 α actively translocates independent of association with ISGF3 γ or of the formation of mature ISGF3. Direct transfer of receptor-activated transcription factor subunits from cytoplasm to nucleus may explain how large numbers of specific responses elicited by different ligands can be accurately and independently regulated in a cell possessing only a limited number of traditional second messenger pathways.

Methods

Gel-shift analysis

Crude extracts were prepared and analyzed by gel retardation using an end-labeled synthetic ISRE oligonucleotide (0.5–5.0 ng) with the sequence GATCCTTCAGTTTCGGTTTCCCTTTCCC (double-stranded), as described previously (Levy et al. 1989) except the quantity of nonspecific DNA in the reaction was reduced to 32 μ g/ml poly[d(I-C)]/poly[d(I-C)] and 8 μ g/ml unrelated oligonucleotide and protein-DNA complexes were resolved on 6% polyacrylamide gels.

Preparation of cytoplasmic ISGF3 γ and ISGF3 α

ISGF3 γ fractions were prepared from S100 extracts of HeLa S3 cells grown in suspension and treated with 1 ng/ml IFN- γ for 18–24 hr, as described (Levy et al. 1990). Protein precipitated by 50% saturated ammonium sulfate was chromatographed on heparin-Sepharose, Phenyl Sepharose, and FPLC Mono Q. Protein that bound to column matrices was eluted with linear KCl gradients or, for Phenyl Sepharose, buffer containing 50% ethylene glycol. Column fractions were assayed by *in vitro* complementation assays. The specific activity of ISGF3 γ was increased ~2000-fold over crude cytoplasm.

ISGF3 α fractions were prepared from S100 extracts of HeLa S3 cells as above, except that cells were treated with 10 mM NaF for 1 hr and IFN- α at 5 ng/ml for 15 min prior to harvest. Protein was alkylated with 10 mM NEM for 10 min at room temperature, and then unreacted NEM was quenched by the addition of 15 mM DTT. Protein precipitated by 30% saturated ammonium sulfate was chromatographed on DEAE-Sepharose, FPLC Mono Q, and FPLC Mono S columns. The specific activity of ISGF3 α was increased 1000- to 2000-fold over crude cytoplasm.

Purification of nuclear ISGF3 by ion-exchange and oligonucleotide chromatography from IFN- γ - plus IFN- α -treated HeLa cells will be described elsewhere (X.-Y. Fu et al., *in prep.*).

Sedimentation analysis

Molecular weight standards or protein fractions (0.2–0.3 ml), adjusted to 10% glycerol and 300 mM KCl, were layered on

12-ml gradients [15–30% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF] and centrifuged for 40 hr at 40,000 rpm and 4°C (SW40 rotor). Fractions were assayed for ISGF3 activity by gel shift. ISGF3 α and ISGF3 γ activities were detected by in vitro complementation of fractions with enriched fractions of ISGF3 γ and ISGF3 α , respectively. Marker fractions were resolved on 10% SDS-PAGE and stained with Coomassie blue. For analysis of the protein composition of gradient fractions, each fraction was concentrated by ultrafiltration, and recovered protein was resolved on 7.5% SDS-PAGE and stained with silver [Wray et al. 1981].

For sedimentation analysis of ISGF3–ISRE complexes, a standard DNA-binding reaction was increased 15-fold using either ISRE or unrelated oligonucleotides. Each binding reaction was loaded onto gradients as described above, except centrifugation was for 24 hr. Fractions were assayed directly on low ionic strength polyacrylamide gels or assayed by in vitro complementation and gel-shift analysis.

Dissociation rate determination

Dissociation rates of protein–DNA complexes were determined essentially as described [Hardy and Shenk 1989; Neill et al. 1990], using enriched ISGF3 α , ISGF3 γ , and nuclear ISGF3 fractions. Formation of DNA–protein complexes reached equilibrium by 20 min [Levy et al. 1989], at which time a 500-fold molar excess of unlabeled ISRE oligonucleotide was added. At serial time points following addition of competitor, aliquots were removed and analyzed by gel shift. The intensity of bands was quantitated by laser densitometry (LKB Ultrascan XL) of autoradiographs exposed without intensifying screens.

Renaturation of ISGF3 γ

A 2000-fold-enriched ISGF3 γ fraction was concentrated by ultrafiltration and resolved on 10% SDS-PAGE prerun with 0.1 mM sodium thioglycolate. Gel regions corresponding to increasing molecular weight proteins were excised, and protein was recovered by elution, acetone precipitation, and renaturation as described [Briggs et al. 1986]. Pellets were resuspended in 40 μ l of buffer containing 100 mM KCl, 0.1% NP-40, and 6 M guanidine-HCl and incubated for 40 min at room temperature. Guanidine-HCl was removed by filtration through G-25 spin columns equilibrated in buffer containing 100 mM KCl and 0.1% NP-40. Following an additional 2-hr incubation at room temperature, ISGF3 γ activity was assayed by in vitro complementation by using partially purified ISGF3 α .

Photoaffinity cross-linking and preparative gel shift

A uniformly 32 P-labeled ISRE oligonucleotide, in which 5-azido-deoxyuridine (a gift from Dr. R.K. Evans) was substituted for thymidine, was incubated with fractions under standard conditions, and DNA–protein complexes were resolved by gel-shift assay. The gel was exposed to 254-nm ultraviolet irradiation for 5 min on ice in a Stratilinker 2400 (Stratagene) and autoradiographed. Gel slices containing protein–DNA complexes were excised, incubated for 15 min at 65°C in SDS sample buffer, and rinsed twice with stacking buffer before electrophoresis into 8% SDS-PAGE [Laemmli 1970].

Preparative gel-shift reactions contained 175 ng of ISRE or unrelated oligonucleotide and $\sim$ 0.5 μ g of a highly enriched fraction of nuclear ISGF3. Following fractionation by standard gel-shift analysis, specific ISRE complexes or analogous regions from control lanes were excised and processed for SDS gel analysis and stained with silver.

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